Nitrate-induced early transcriptional changes during imbibition in non-after-ripened *Sisymbrium officinale* seeds

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We have here demonstrated for the first time that nitrate not only accelerates testa rupture of non- AR seeds but also modifies expression pattern of the cell-wall remodeling proteins (mannanases; *SoMAN6* and *SoMAN7*) and key genes belonging to metabolism and signaling of ABA (*SoNCED6*, *SoNCED9*, *SoCYP707A2* and *SoABI5*) and GAs (*SoGA3ox*, *SoGA20ox*, *SoGA2ox* and *SoRGL2*). These results were obtained during *Sisymbrium officinale* seed imbibition in the absence of endosperm rupture. Exogenous ABA induced a notable inhibition of testa rupture in both absence and presence of nitrate being this effect sharply reversed by GA_{4+7}. However, nitrate was capable to provoke testa rupture in absence of ABA synthesis. The expression of *SoMAN6* and *SoMAN7* were positively altered by nitrate. Although ABA synthesis seems apparent at the start of non-AR seed imbibition, taken together the results of *SoNCED6*, *SoNCED9* and *SoCYP707A2* expression seem to suggest that nitrate leads to a strong net ABA decrease. Likewise, nitrate positively affected the *SoABI5* expression when the *SoNCED9* expression was also stimulated. By contrast, at the early and final of imbibition, nitrate clearly inhibited the *SoABI5* expression. The expression of *SoGA2ox6* and *SoGA3ox2* are strongly inhibited by nitrate whereas of *SoGA20ox6* was stimulated. On the other hand, *SoRGL2* transcript level decreased in the presence of nitrate. Taken together, the results presented here suggest that the nitrate signaling is already operative during the non-AR *S. officinale* seeds imbibition. The nitrate, in cross-talk with the AR network likely increases the favorable molecular conditions that trigger germination.

Introduction

The germination of a non-dormant (ND) seed is a key phase in the life cycle of higher plants, because it starts the regeneration of the mother plant (Hilhorst et al. 2010, Nonogaki et al. 2010). The appearance of ND seeds depends on environmental cues such as low temperatures, light or after-ripening (AR), which exert a constant cross-talk with plant hormones (Kucera et al. 2005, Cadman et al. 2006, Carrera et al. 2008, Bentsink and Koornneef 2008, Holdsworth et al. 2008a, Iglesias-Fernández et al. 2011a, Penfield and Springthorpe 2012). Thus, cold or light cause degradation of certain inhibitory

Abbreviations – ABA, abscisic acid; ABI5, TF bZIP type; AR, after-ripening; GAs, gibberellins; NCED, 9-*cis*-epoxy-carotenoid dioxygenase; ND, non-dormant; PB, paclobutrazol; RGL2, RGA-LIKE2 DELLA protein; RT-qPCR, real-time semi-quantitative PCR; TF, transcription factor.

transcription factors (TFs) leading to an increase in gibberellins (GAs) synthesis and an inhibition of DELLA proteins and abscisic acid (ABA) action (Achard and Genschik 2009, Fleet and Williams 2011, Hedden and Thomas 2012). In order to conduct a detailed and comprehensive study, the germination has been divided into two sequential phases: imbibition and radicle emergence (Weitbrecht et al. 2011). The early phase of imbibition (i.e. phase I; Finch-Savage and Leubner-Metzger 2006), which takes place in both dormant and ND seeds, has been scarcely studied at molecular level. In some Brassicaceae as *Arabidopsis*, *Lepidium* and *Sisymbrium officinale* (Müller et al. 2006, Iglesias-Fernández and Matilla 2010) and Solanaceae as tobacco (Petruzzelli et al. 2003), germination has two separate and visible stages. That is, the testa rupture which takes place during imbibition phase II (Müller et al. 2006) and the radicle elongation that completes germination by rupturing the softened micropylar endosperm layer which covers it (starting phase III). The high transcriptomic and proteomic activities that take place during imbibition are tightly coordinated by hormonal networks such as ABA and GAs (Holdsworth et al. 2008a, Iglesias-Fernández and Matilla 2009, Morris et al. 2011, Weitbrecht et al. 2011). GAs and ABA metabolism and sensitivity are oppositely regulated in seeds (Yamaguchi 2008, Nambara et al. 2010). Consequently, a cross-talk between both signaling pathways and a tight control of GAs/ABA ratio must exist during early germination (Kucera et al. 2005, Feurtado and Kermode 2007). That is, GAs action appears to be essential to recover the signaling network that irreversibly leads to the radicle emergence (Yamaguchi 2008, Fleet and Williams 2011, Hedden and Thomas 2012).

The synthesis of active *cis*(+)-S-ABA takes place in the cytoplasm in close collaboration with the plastid (Rodríguez-Gacio et al. 2009). In short, the ABA precursors are synthesized in the plastids and the *cis*-xanthophylls are cleaved by a family of 9-*cis*-epoxycarotenoid dioxygenases (NCED) to form the first ABA-precursor, *cis*-xanthoxin. Xanthoxin is moved to the cytosol and converted to ABA-aldehyde, which is oxidized to *cis*(+)-S-ABA by an ABA-aldehyde oxidase. The active ABA is inactivated by an ABA-8′-hydroxylase (member of the *CYP707A* subfamily) and can be finally converted to phaseic and dihydro-phaseic acids. In this ABA metabolic pathway, the *NCED* and *CYP707A* gene families are the key points of ABA synthesis, inactivation and regulation (Saito et al. 2004, Rodríguez-Gacio et al. 2009, Nambara et al. 2010, Weitbrecht et al. 2011). During early imbibition of ND seeds, a net decrease in endogenous ABA was observed (Preston et al. 2009). The CYP707A synthesis might contribute to this ABA decrease (Liu et al. 2009). The *CYP707A* genes are expressed in specific parts of the seed (i.e. micropylar endosperm and coleorhiza; Millar et al. 2006, Okamoto et al. 2006, Toh et al. 2008). However, *de novo* ABA synthesis is necessary to maintain the dormancy and trigger germination through a cross-talk with GAs and other germination cues (Okamoto et al. 2006, Seo et al. 2006, Toh et al. 2008, Preston et al. 2009, Nambara et al. 2010, Weitbrecht et al. 2011). Genetic analyses reveal that ABI3 (B3 type), ABI4 (AP2 type) and ABI5 (bZIP type) are key TF that confer ABA responsiveness (Finkelstein et al. 2002, Holdsworth et al. 2008a). In imbibed *Arabidopsis* seeds, ABI5 is regulated by ABA, being an indicator of ABA response (López-Molina et al. 2001, Piskurewicz et al. 2009). By contrast, the dormancy loss of many seeds is directly related to the increase in GAs sensitivity and is known for its antagonistic role with ABA (Koornneef et al. 2002, Kucera et al. 2005, Feurtado and Kermode 2007, Nonogaki et al. 2010, Iglesias-Fernández et al. 2011a, Weitbrecht et al. 2011). On the other hand, the DELLA protein RGL2 (*RGA-LIKE2*) is needed to control ABI5 function and ABA levels. RGL2 and ABI5 are positive and negatively regulated by ABA and GAs, respectively. ABI5 acts as a key point during the germination process (Piskurewicz et al. 2008). Shortly, in the absence of GAs, RGL2 inhibits germination by promoting ABA synthesis and signaling, and ABA itself stabilizes DELLAs (Fleet and Williams 2011).

The radicle emergence in *S. officinale* ND seeds is sharply accelerated by the presence of nitrate in the germination medium (Hilhorst and Toorop 1997, Iglesias-Fernández et al. 2007, Iglesias-Fernández and Matilla 2009). Nitrate is known to promote the *Arabidopsis* germination acting together with cold and light (Alboresi et al. 2005, Finch-Savage et al. 2007). However, the mechanism of nitrate action remains unsolved and it is debated whether it always participates in dormancy release and/or germination induction or it only affects some seeds (i.e. seeds of nitrophilous plants). On the other hand, it is also unknown whether nitrate has some effect during dormant-seeds imbibition. Due to the lack of physiological and molecular knowledge about the imbibition responses during phase I and II, a molecular study in the presence of nitrate will provide a useful tool. In this work, we demonstrate that nitrate not only positively alters the testa rupture in non-AR *S. officinale* seeds but also varies the expression pattern of mannanases (i.e. *SoMAN6* and *SoMAN7*) and key genes belonging to metabolism and signaling of ABA (i.e. *SoNCED6*, *SoNCED9*, *SoCYP707A2* and *SoABI5*) and GAs (i.e. *SoGA3ox*, *SoGA20ox*, *SoGA2ox* and *SoRGL2*). Likewise, it is also considered the possibility

that at some points the nitrate possesses an independent signaling of ABA and GAs.

Materials and methods

Plant materials

Fruits of wild hedge mustard (*S. officinale*) were always collected in the Campus of Santiago de Compostela University (Santiago de Compostela, Galicia, northwest Spain) throughout July and August 2011. After the fruits were harvested, they were dried at room temperature for 1 month and the seeds were separated from the rest of the fruit (i.e. replum, valves and pedicel). The mature dark seeds were isolated from the light ones. The light seeds were less mature than the dark ones and their germination rate is also minor (Iglesias-Fernández et al. 2007).

Determination of imbibition phases I, II and III

The imbibition curves were carried out with 50 dry and viable seeds (three replicates) under the same germination conditions described below. Fresh weight of the seeds was evaluated until radicle emergence had occurred.

Testa rupture and germination assays

Three replicates of 50 seeds were sown in 90 mm Petri dishes on two layers of filter paper (Whatman No.1) and one membrane filter (mixed cellulose ester) moistened with 3 ml of sterile water or 20 m*M* KNO_3 pH 7.0 both supplemented with solutions of 100 μ*M* GAs_{4+7} (Duchefa Biochemie, The Netherlands), inhibitor of GAs synthesis [25 μ*M* paclobutrazol (PB), Sigma Aldrich, Spain] or 100 μ*M* ABA (Sigma Aldrich, Spain). Germination assays take place in a growth chamber at 23°C, under long-day conditions (16 h light/8 h darkness; 55 μmol m^{-2} s^{-1} of photosynthetic photon flux density). Seeds were not surface-sterilized for the purpose of avoiding influencing their dormancy status; however fungal infections were not detected by light microscopy. The testa breakage did not occur in the micropylar region but below this area. Seeds were scored as germinated when the radicle emergence through the seed-coats was visible under magnifying lens. Germination test were performed in three biological samples using three technical replicates.

Light microscopy

In order to observe the alterations in the lateral and micropylar single-layer endosperm of *S. officinale* seeds, dry and 24 h imbibed seeds with and without testa rupture were fixed in 50 m*M* sodium phosphate buffer, pH 6.8, containing 2% (w/v) *p*-formaldehyde (Panreac Química, Barcelona, Spain) and 2% (v/v) glutaraldehyde (Merck, Germany) for 2 days at 4°C. The histological features were done in at least six seeds. Other light microscopy characteristics were as previously described (Iglesias-Fernández and Matilla 2010).

RNA isolation and cDNA synthesis

Seeds were imbibed for 3, 6, 12, 22 and 26 h in the above germination conditions and three replicates of 50 seeds for each time point were collected in 2 ml tubes being immediately frozen in liquid N_2 and stored at −80°C till RNA extraction. A grinding ball (stainless steel 0.7 mm) was add to the tubes and seeds were finely pulverized using a MiKro-Dismembrator-S (Sartorius AG, Goettingen, Germany) for 2 min at 170 *g*. Total RNA was obtained by using the SV Total RNA Isolation System (Promega, Madison, WI). The purity of RNA was analyzed spectrophotometrically measuring A_{260} and A_{280}. The RNA samples were kept at −80°C until used. The cDNA was synthesized from 0.1 mg of total RNA using the First-Strand Synthesis kit for RT-PCR (Roche® Diagnostics, Mannheim, Germany) with oligo-dT as a primer according to the manufacturer's directions. Samples were stored at −20°C for further analysis.

Isolation of *SoNCED6*, *SoNCED9*, *SoCYP707A2*, *SoRGL2*, *SoABI5*, *SoMAN7* and *SoMAN9* partial-length cDNA

The cDNA sequences were obtained from RNA seeds using degenerate primers pairs based on highly conserved regions of the corresponding genes in other species and available in the database (Table 1). PCR reactions were performed in the following conditions: 95°C for 5 min, 40 cycles of 95°C for 45 s, 45–54°C for 45 s, 72°C for 1 min and a final elongation step of 7 min at 72°C. PCR reactions took place in a 25 μl using puRe Taq Ready-To-Go PCR Beads (each bead contain 200 μ*M* of each dNTP, 10 m*M* Tris–HCl, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 2.5 units of puRe Taq DNA polymerase and reaction buffer). The beads were rehydrated in presence of 21 μl of sterilized water, 1 μl of 5 μ*M* reverse primer (final concentration 300 n*M*) and 1 μl of 5 μ*M* forward primer (final concentration 300 n*M*) and 1 μl of cDNA was added. PCR products were analyzed electrophoretically and extracted from the agarose gel using MiniElute™ Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced for confirmation. Sequences were compared with existing sequences in target databases utilizing BLAST (Altschul et al. 1997). They possess the

Table 1. List of degenerate primers used for PCR assays in the isolation of partial length cDNAs.

Gene	Primer name	Primer sequence (5′-3′)
SoCYP7 07A2	*FwCYP707A2*	ATGATWGGVCCAGARGCHMT
	RvCYP707A2	GTRGTGAGRTGRTGNACDAG
SoNCED9	*FwNCED9*	TMTTYGACGGYGAYGGNATG
	RvNCED9	HGTWCCATGRAAWCCATAHGG
SoNCED6	*FwNCED6*	CRCTTAAYTCAAYCCGGGTYGG
	RvNCED6	GCTCAAACCAACSYAYCCRAAC TTAAACC
SoABI5	*FwABI5*	GGNGAGATGACDYTKGAGGAT
	RvABI5	GACTCDCKRTTCTTDATCAT
SoRGL2	*FwRGL2*	ATGAAGAGAGGNTAYGGNGA AACATG
	RvRGL2	GCNGTRAARTGNGCRAAYTTNAR RTANGGRCA
SoMAN6	*FwMAN6*	TWTGGNGGVAARGCWCARTA
	RvMAN6	GAACMGARGCAAAATCAA
SoMAN7	*FwMAN7*	TTYMAGGGDTTGGATTTTGY
	RvMAN7	YTGTGCRTCTTGRATGTGD

Table 2. List of primers used for the RT-PCR assays.

Gene	Primer name	S Primer sequence (5′-3′)	Amplicon size (bp)
SoCYP 707A2	*FwCYP707A2*	AGTAGGAGTGAAGGAGGAGG	99
	RvCYP707A2	CGATGATGTTGTCAGCGA	
SoNCED9	*FwNCED9*	GTCGGTCTGTTTTCCCCA	86
	RvNCED9	ATAGACCTCGGGCATTGAAG	
SoNCED6	*FwNCED6*	TTCGTACCGGAGGAAGGAGG	116
	RvNCED6	GCGACTTGCTTCATCTCCGA	
SoABI5	*FwABI5*	GTACCACCTATTCAGCCAGGT	117
	RvABI5	ACTATGTCCTAGCCCGTCTGAC	
SoRGL2	*FwRGL2*	AAGATGGCGGATGACGG	117
	RvRGL2	GGACAGAACCATCTCAAGC	
SoMAN6	*FwMAN6*	CCCACCCTCAGAGACTTC	133
	RvMAN6	TCACTCTATTCAGCACCGT	
SoMAN7	*FwMAN7*	CCGAACCATTCAGGCTTG	126
	RvMAN7	CCTCACCTCAAAGCAAGACTC	
18S RNA	*Fw 18S-RNA*	GGCTCGAAGACGATCAGATA	87
	Rv 18S-RNA	TCATAAGGTGCCGGCGGAGT	

partial-length cDNA of genes with high similarity to *NCED6*, *NCED9*, *CYP707A2*, *RGL2*, *MAN6*, *MAN7* and *ABI5* genes of other plant species (GenBank, databases). These sequences were named as follows: *SoNCED6*, *SoNCED9*, *SoCYP707A2*, *SoRGL2*, *SoMAN6*, *SoMAN7* and *SoABI5* and registered in GenBank under the accession numbers: *SoNCED6* (JQ955595), *SoNCED9* (JQ922419), *SoCYP707A2* (JQ955596), *SoRGL2* (JQ955594), *SoABI5* (JQ955593), *SoMAN6* (JX113231) and *SoMAN7* (JX113232). Sequences for *SoGA20ox2*, *SoGA2ox6* and *SoGA3ox2* were previously determined and registered (Iglesias-Fernández and Matilla 2009).

Semi-quantitative real-time polymerase chain reaction assays

The RT-qPCR was performed using first-strand cDNA as template in an iCycleriQ™ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA) and iQ SYBR Green dye (Bio-Rad Laboratories) was used to detect the PCR products. The reaction was conducted using the gene-specific primers synthesized from the sequences obtained for *SoMAN6*, *SoMAN7*, *SoNCED*, *SoNCED9*, *SoCYP707A*, *SoRGL2* and *SoABI5* (Table 2). Moreover, the 18S-RNA was used as a control because of the fact that it was found to be expressed at constant levels throughout the study period. For each 25 µl reaction, 1 µl of cDNA sample was mixed with 12.5 µl of iQ™ SYBR Green® Supermix (Bio-Rad Laboratories), 0.5–1 µl of 12 µ*M* each primer (final concentration 240 n*M*)and sterile water up to final volume. Samples were subjected to thermal cycling conditions of DNA polymerase activation at 95°C for 4 min, 40 cycles of 95°C for 45 s, 45 s at 48°C (for *SoMAN6*), 52°C (for *SoGA20ox2* and *SoGA2ox6*), 53°C (for *SoCYP707A*), 55°C (for *SoRGL2* and *SoGAS3ox2*), 57°C (for *SoMAN7*) and 64°C (for *SoNCED9* and *SoABI5*); 72°C for 45 s and a final elongation step of 7 min at 72°C was performed. The melting curve was designed to increase 0.5°C every 10 s from 62°C to 74°C (depending on the gene). These analyses were performed with three different cDNAs (from three different RNA) for each time-point. The amplicon was analyzed by electrophoresis and sequenced for confirmation. Real-time PCR efficiency was estimated via calibration dilution curve and slope calculation. Expression levels were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of the PCR reaction (CT), the ΔΔCT method was used to analyze data (Pfaffl 2001). The expression in dry seeds was used to relativize data and determine the transcripts levels modifications (Finch-Savage et al. 2007). For statistical analysis of gene-expression data a Student's *t*-test was performed between nitrate-treated seeds vs non-treated seeds.

Results

Testa rupture and germination of *S. officinale* non-AR seeds

The rupture of the testa and endosperm are separate events in the germination of *S. officinale* (i.e. two-step germination; Fig. 1) and they are dependent of the presence of nitrate (KNO_3) in the germination medium. The results of the ruptures in the presence of distilled water (control) and nitrate (in brackets) indicated that the testa rupture began at 13 h imbibition and reached 2 ± 1

Fig. 1. Different phases of *Sisymbrium officinale* seeds germination. (A) Dry seed, (B) imbibed seed in phase I, (C) imbibed seed starting the testa rupture (phase II) and (D) germinated seed (phase III).

(10 ± 1), 6 ± 1(20 ± 1), 35 ± 3 (71 ± 2) and 75 ± 3% (89 ± 1%) at 15, 22, 35 and 60 h, respectively (Fig. 2A). That is, while the time to reach 50% testa rupture was 40 h for the control, it was only 26 h for nitrate. On the other hand, the beginning of the endosperm rupture takes place from 27 h, with data values of 2 ± 1 (3 ± 1), 4 ± 1 (25 ± 3), 13 ± 1 (42 ± 4) and 30 ± 4% (50 ± 5%) at 30, 38, 42 and 48 h, respectively (Fig. 2B). Unlike testa rupture, the 50% endosperm rupture occurs at 51 and 48 h for the control and nitrate, respectively. In short, the nitrate markedly stimulated the testa and the endosperm ruptures between 28–38 and 35–50 h, respectively. Maxima testa and endosperm ruptures (100%) in the presence of nitrate were achieved at 6 days in non-AR *S. officinale* (Fig. 2A, B). On the other hand, the imbibition curve of seeds displayed the common triphasic model of water uptake [phases I, II and III; Fig. 2A (inset)]. In phase II, (1) the cell appearance of both lateral endosperm and cotyledon below broken testa and that of micropylar endosperm are similar (i.e. empty protein bodies) and (2) the depletion of protein bodies was also clearly observed in lateral sub-apical zone of imbibing radicle (Fig. 3). The presence of nitrate in imbibition medium does not alter this pattern (data not shown).

Effect of ABA and GAs on testa rupture in non-AR seeds

The presence of nitrate stimulated almost six times the testa rupture percentage with respect to the control (Fig. 2A). Exogenous ABA inhibited the percentage of testa rupture by about 50% in the control and caused an inhibition of 60% in presence of nitrate (Table 3). The addition of GA_{4+7} to the germination medium almost duplicated the testa rupture in the presence and absence of nitrate; whereas the addition of ABA and GA_{4+7} together produced an inhibition of around 50% in both control and nitrate. Interestingly, in the presence of PB the percentage of testa rupture was strongly inhibited

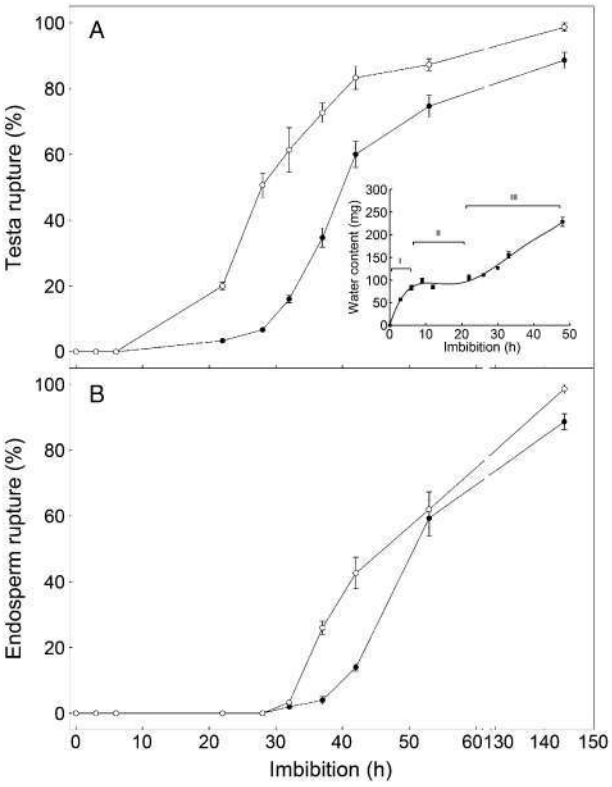

Fig. 2. Time-course of testa (A) and endosperm (B) rupture in the presence (open circles) and absence (closed circles) of nitrate. Inset: water uptake phases. Data are means ± SE of the five to six independent experiments.

in the absence of nitrate but it was accelerated in its presence (Table 3).

Alterations promoted by nitrate in the expression of *SoMAN6* and *SoMAN7* during early imbibition

The *SoMAN6* expression, which is quantitatively lower than that of*SoMAN7*, increased progressively in the absence of nitrate (control) being at 26 h twice times more with respect to 3 h (Fig. 4A). In the presence of nitrate the *SoMAN6* expression was markedly altered. Thus, nitrate stimulated this expression at very early phase of imbibition and strongly inhibited it between 22 and 26 h, being at 26 h half than that *SoMAN6* expression in the control (Fig. 4A). As well as in *SoMAN6*, the *SoMAN7* expression was also detected in dry viable seeds. In the control there was a sharp stimulation of *SoMAN7* expression during the first 6 h imbibition and then it gently increased. The presence of nitrate markedly inhibited its expression at 3 h. The *SoMAN7* expression pattern was not altered by presence of nitrate between 6 and 26 h (Fig. 4B). In brief, when testa is broken but micropylar endosperm is not,

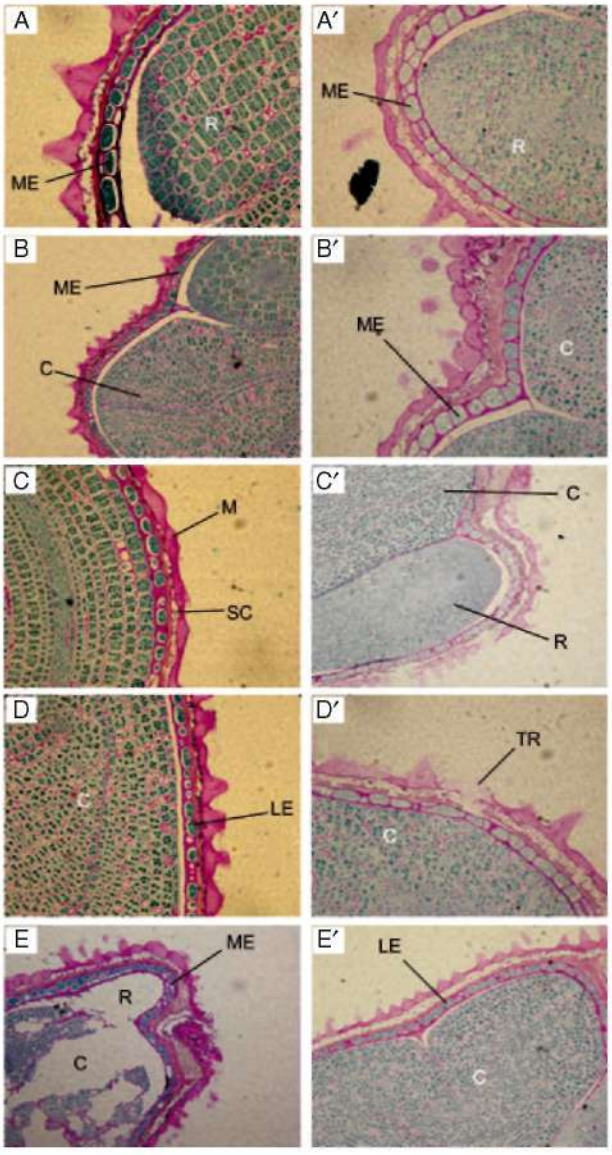

Fig. 3. Structure of a dry mature seed (A–D), died seed (E) and imbibed mature *Sisymbrium officinale* seed (A′–D′ and F). C, cotyledon; LE, lateral endosperm; M, mucilage; ME, micropylar endosperm with filled (A, B, E) and empty protein bodies (A′, B′); R, radicle; SC, seed coat; TR, zone of testa rupture. Experiments were carried out using at least 20–25 seeds. Staining data are shown in section Materials and methods.

the results of the Fig. 4A, B suggested that *SoMAN6* and *SoMAN7* appears to be temporally involved in the imbibition process. In the absence of nitrate, the *SoMAN6* expression was markedly reduced in AR seeds. However, the presence of nitrate caused a strong stimulation of *SoMAN6* expression. This effect was not observed with the *SoMAN7* expression (data not shown).

Table 3. Effect of ABA and GAs (GA_{4+7}) on seed coat breakage percentage in non-AR *Sisymbrium officinale* seeds treated during 26 h with NO$_3$K. PB, paclobutrazol; [a]Treatments in water; [b]treatments in NO$_3$K. [*]Data are mean values of three replicates ± SE.

Treatment	Seed coat breakage (%)[*]
H_2O[a]	10 ± 2
NO_3K[b]	57 ± 3
ABA[a]	4 ± 2
ABA[b]	35 ± 1
GA_{4+7}[a]	21 ± 3
GA_{4+7}[b]	90 ± 2
(GA_{4+7} + ABA)[a]	13 ± 1
(GA_{4+7} + ABA)[b]	55 ± 3
PB[a]	1 ± 1
PB[b]	50 ± 1

Nitrate alters *SoNCED6*, *SoNCED9*, *SoCYP707A2* and *SoABI5* expression patterns during early imbibition

The expression of two members of NCED family (i.e. *SoNCED6* and *SoNCED9*) was studied in *S. officinale* non-AR seeds (Fig. 5). In the control *SoNCED6* expression increased until 6 h (phase I), gently decreasing between 12 and 26 h (phase II). By contrast, the nitrate provoked a sharp stimulation of this expression at 3 h and a very hard inhibition at 22 and 26 h (Fig. 5A). On the other hand, the *SoNCED9* expression in the control was much higher than that of *SoNCED6* and it was markedly stimulated during the early 3 h imbibition subsequently decreasing until 26 h. Nitrate did not affect the *SoNCED9* expression during the first 6 h imbibition. However, between 22 and 26 h it negatively affected the expression (Fig. 5B). Comparing to the expression of *SoNCED6* and *SoNCED9*, the *SoCYP707A2* expression is very high in the nitrate absence/presence; the presence of nitrate increased the gene expression during the first 22 h imbibition (Fig. 5C). With respect to *SoABI5* expression, two opposite effects of nitrate were observed: (1) an inhibition of expression during the first 3 h imbibition and a strong inhibition at the end of phase II (i.e. 26 h) and (2) a stimulation of expression during middle phase of imbibition (i.e. 12 h) that coincides with the lowest expression in the control (Fig. 5D).

SoGA3ox2, *SoGA20ox2*, *SoGA2ox6* and *SoRGL2* expression patterns are modified by nitrate during *S. officinale* early imbibition

The *SoGA3ox2* expression, which reached a noticeable minimum level in the control at 12 h imbibition, was strongly nitrate-inhibited during the first 6 h imbibition and barely detectable. Although the *SoGA3ox* expression increased from 6 to 26 h in the presence of nitrate,

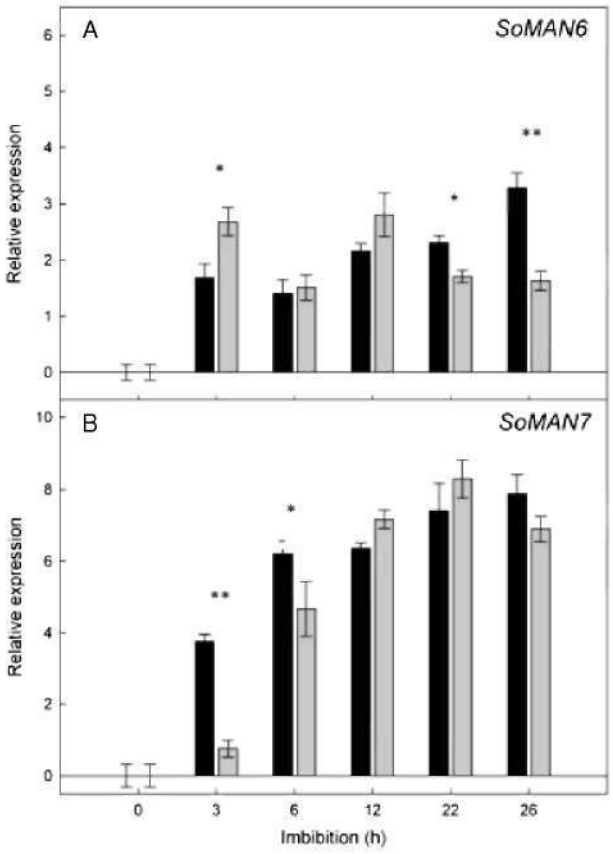

Fig. 4. Transcript analysis by real-time PCR of *SoMAN6* (A) and *SoMAN7* (B) in the absence (black bars) and presence (grey bars) of nitrate during the time course of imbibition of non-AR *Sisymbrium officinale* seeds. Errors bars indicate the SD of three independent experiments. Symbols (*) and (**) indicate significant differences ($P < 0.05$ and $P < 0.01$, respectively) as determined by *t*-test.

it was quantitatively lower than in the control. That is, nitrate was an inhibitor of *SoGA3ox2* expression, except for 12 h (Fig. 6A). The *SoGA20ox2* expression, which was quantitatively more intense than that of *SoGA3ox2*, was only slightly stimulated by nitrate during the first 3 h imbibition and inhibited during the rest of the studied period (Fig. 6C). Finally, the *SoGA2ox6* expression pattern was differently affected by nitrate. Thus, nitrate scarcely affected it during the first 3 h imbibition and strongly inhibited it from 22 to 26 h (Fig. 6B). The *SoRGL2* expression was not altered by nitrate during the first 12 h; but it was inhibited from 22 to 26 h (Fig. 6D). Summarizing, with the exception of *SoGA20ox2* expression at 3 h (stimulated by nitrate), the results of Fig. 6 indicate that the presence of nitrate in the germination medium of dormant *S. officinale* seeds leads to a decrease in the expression of all the genes studied related to the GAs metabolism and signaling.

Discussion

Effect of nitrate on testa rupture

Germination '*sensu stricto*' (i.e. phases I and II) is associated with cellular and molecular events which enable the radicle to emerge (Kucera et al. 2005, Holdsworth et al. 2008a, Nonogaki et al. 2010). Upon completion of phase II, the radicle protrudes through the previously weakened testa and endosperm envelopes (Rodríguez-Gacio et al. 2012). The seeds of *Arabidopsis*, *Lepidium sativum* and *S. officinale* are surrounded by the testa and a single-layer endosperm (Debeaujon and Koornneef 2000, Liu et al. 2005, Iglesias-Fernández and Matilla 2010, Linkies et al. 2010). As in other Brassicaceae, the testa cells of *S. officinale* produce mucilage which positively affects water uptake and germination (Western et al. 2004, Iglesias-Fernández et al. 2007). This mucilage production is not affected by exogenous nitrate (data not shown). Although the testa and the endosperm have a key role in seed dormancy and germination (León-Kloosterziel et al. 1994, Koornneef et al. 2002, Bethke et al. 2007, Nonogaki et al. 2007, Koizumi et al. 2008), the relationship between both covering layers is unknown at present. However, existing information supposes that the endosperm could affect the testa (Haughn and Chaudhury 2005). Before this work, it was shown that rupture of testa and endosperm are two sequential steps during the germination of *S. officinale* seeds (Iglesias-Fernández and Matilla 2010). This temporal separation was also demonstrated in *Arabidopsis thaliana*, *L. sativum* and *Nicotiana tabacum* (Petruzzelli et al. 2003, Kucera et al. 2005, Müller et al. 2006, Piskurewicz et al. 2008). Recently, it was shown in *L. sativum* that following testa rupture, germination is regulated by two opposing forces: the radicle extension and the resistance of the surrounding micropylar endosperm cap, which progressively declines through autolysis (Morris et al. 2011). To gain inside into the mechanism that operates in *S. officinale* seeds before and during the testa rupture, we have used dimbibed non-AR seeds with testa rupture and absence of micropylar endosperm rupture (Fig. 1). The testa rupture initially occurs in a single slit close to the micropylar area (Figs 1C and 3). This indicates that there must be a specific area in the testa with lower resistance to breakage. In this scenario, the presence of nitrate accelerates the testa rupture barely affecting the radicle emergence. Nevertheless, we have not yet been able to find an increase in nitrate-induced cell elongation below the zone of testa rupture. If so, it could serve to suggest that the modifications in the turgor of neighboring cells would be involved in the mechanism of the testa rupture. However, as the pressure generated by imbibing radicle

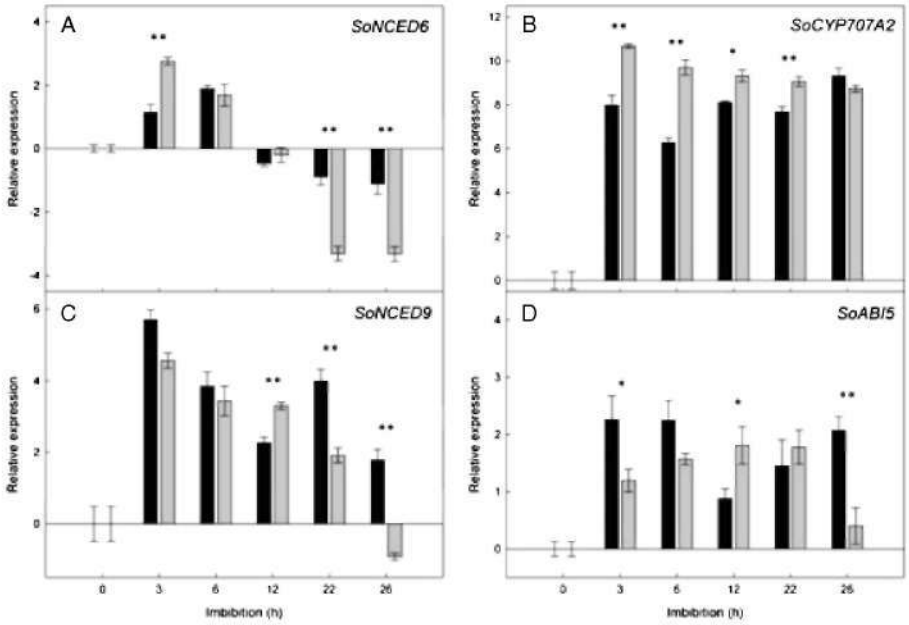

Fig. 5. Transcript analysis by real-time PCR of *SoNCED6* (A), *SoNCED9* (C), *SoCYP707A2* (B) and *SoABI5* (D) in the absence (black bars) and presence (grey bars) of nitrate during the time course of imbibition of non-AR *Sisymbrium officinale* seeds. Errors bars indicate the SD of three independent experiments. Symbols (*) and (**) indicate significant differences ($P < 0.05$ and $P < 0.01$, respectively) as determined by *t*-test.

is involved in the seed envelopes rupture (Nonogaki et al. 2010, Linkies et al. 2010), the radicle must initially cause a force to push-out and break the previously softened micropylar endosperm and consequently the testa shattering. Contrary to this, the micropylar endosperm of hard seeds appeared to be predisposed to radicle emergence because its cells were smaller (thin cell walls) than those of the lateral endosperm (Gong et al. 2005). Recently, it was demonstrated in *Genipa americana* (Rubiaceae) seeds that embryos displayed an ABA inhibited growth prior to radicle protrusion (Queiroz et al. 2012). Similar observations were previously obtained in coffee seeds (da Silva et al. 2008). Taking these results together, it is possible that the force threshold required to break each two covering layers was different, or alternatively it may be that the testa and endosperm are sequentially broken by the same pushing force because the testa is previously softened. At present, the existence of a mechanism to weaken the testa is unknown. However, if the weakening of the seed endosperm (i.e. *Lepidium*; Weitbrecht et al. 2011) occurs prior to its rupture, the testa could also possess a temporal breakening. It is worth experimenting on it.

Interestingly, non-AR *S. officinale* seeds imbibed more slowly than the AR ones and this behavior is temperature-dependent (Iglesias-Fernández and Matilla 2009). During *Arabidopsis* imbibition, a local increase in seed size in the proximity of micropylar area was observed; this event is also observed in tomato (Joosen et al. 2010). It is unknown if there are pre-determined breaking points in the testa before its rupture starts. The tobacco testa rupture begins close to the micropyle extending on the testa along the ridges (Leubner-Metzger 2003). Progression of testa rupture is facilitated by channel-like structures subjacent to ridges, suggesting the existence of predetermined breaking zones. On the other hand, in the imbibition phase I and II of *S. officinale*, notable alterations in the lateral and micropylar endosperm cells were histologically demonstrated (i.e. degradation of organelles as proteic bodies; Fig. 3) and the testa rupture is spatially localized, it would not be surprising that the programmed cell death (PCD) would be involved in this dismantling process designed to allow the protrusion. In the lateral endosperm of *Solanum lycopersicum* seeds, PCD is influenced by embryo signals such as GAs (DeBono and Greenwood 2006). This PCD is similar to that of maize (Gunawardena et al. 2001), wheat (Domínguez et al. 2004) and castor seeds (Greenwood et al. 2005).

In this work, we have demonstrated that the positive effect of nitrate on testa breakening is affected by exogenous ABA and GA_{4+7}. Both hormones quantitatively affected the same in the control and nitrate-treated seeds. Perhaps, the nitrate has its own signaling which is fully or partially independent of those of ABA and GAs. These possibilities need to be studied. Related to this

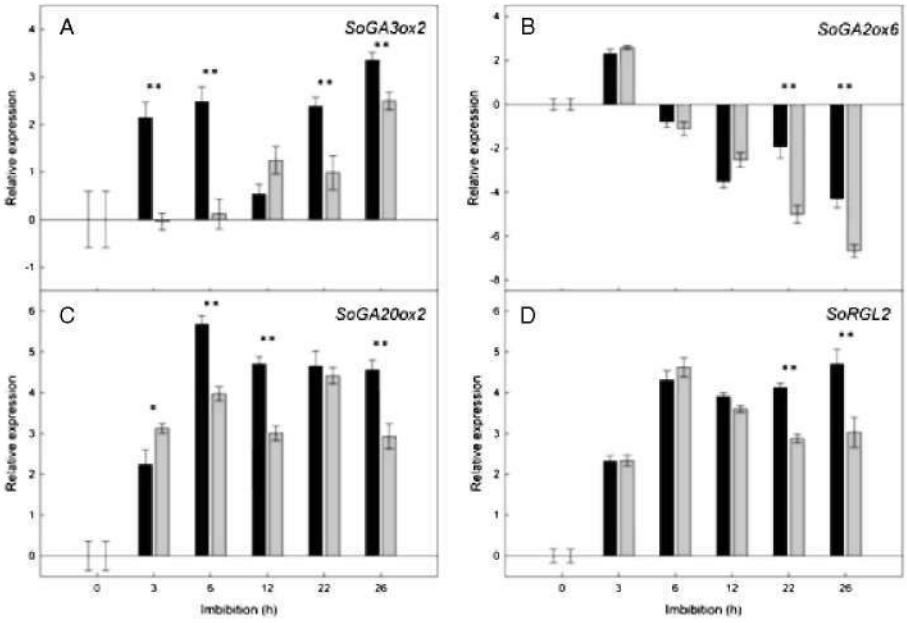

Fig. 6. Transcript analysis by real-time PCR of *SoGA3ox2* (A), *SoGA20ox2* (C), *SoGA2ox6* (B) and *SoRGL2* (D) in the absence (black bars) and presence (grey bars) of nitrate during the time course of imbibition of non-AR *Sisymbrium officinale* seeds. Errors bars indicate the SD of three independent experiments. Symbols (*) and (**) indicate significant differences ($P < 0.05$ and $P < 0.01$, respectively) as determined by *t*-test.

course, the results of testa rupture in presence of nitrate and absence of GAs synthesis (i.e. PB in the germination medium) indicated that this break was nitrate-induced (Table 3). In ND seeds, the application of ABA inhibits the passage between phase II and III and also the late cell expansion in the embryo, but does not affect phases I and II and testa breakage (da Silva et al. 2004, Manz et al. 2005, Müller et al. 2006). Endosperm rupture, but not testa rupture, is inhibited by ABA in *Arabidopsis* and *Lepidium* and counteracted by GAs (Koornneef et al. 2002, Yamaguchi and Kamiya 2002, Leubner-Metzger 2003, Kucera et al. 2005). It is also verified in mutants that are impaired in ABA degradation such as cyp707a1 and cyp707a2 (Okamoto et al. 2006). In this work, we have observed that ABA induces a notable inhibition of testa rupture both in the control and in the presence of nitrate being this inhibitory effect reversed by GA_{4+7} (Table 3). An increase in embryo growth potential followed by cell extension is promoted by GAs and inhibited by ABA (Nonogaki 2006, Da Silva et al. 2008, Holdsworth et al. 2008a).

The *SoMAN6* and *SoMAN7* expression is nitrate-affected

It is well established that reduced dormancy in testa mutants of *A. thaliana* was caused by alterations in the testa structure (Debeaujon and Koornneef 2000, Koornneef et al. 2002, Rajjou et al. 2004, Hilhorst et al. 2010). The data of this work demonstrated that the expression of *SoMAN6* and *SoMAN7* changed during phase I. Previously, we have demonstrated that the MAN activity: (1) was barely detected in non-AR seeds and increased during phases I and II, (2) dramatically rose in dry AR seeds and during the first 3 h of phase I and (3) it was up-regulated by both GAs and ethylene (Iglesias-Fernández and Matilla 2009). Taken together these evidence and those included in a recent MAN review (Rodríguez-Gacio et al. 2012), we can argue that the MANs are related to the *S. officinale* germination. Recently, we suggested that *AtMAN5*, *AtMAN7* and specially *AtMAN6* are important in *A. thaliana* germination, facilitating the hydrolysis of the mannan-rich endosperm cell walls (Iglesias-Fernández et al. 2011b).

A recent work on the loss of seed dormancy suggested that some hydrolytic enzymes, facilitating testa rupture, might be released by the endosperm and/or the radicle (Hilhorst et al. 2010). Although in the seeds used in this work the testa rupture is the most visible characteristic, we cannot rule out that MAN may have some important role in the early preparation of the micropylar endosperm softening and its nearest areas. Results in *Lepidium* demonstrated that during early imbibition (2–5 h) an embryo signal is necessary and sufficient to induce the endosperm weakening (Müller et al. 2006). This fact was also found in *Arabidopsis* (Ogawa et al. 2003, Yamauchi et al. 2004). Nevertheless, our data do not appear to be

directly related to the variations between MAN expression and the testa rupture. Testa is a complex cover consisting of several layers of cells whose molecular participation in germination, either independently or hormonally coordinated with the endosperm and/or embryo, is still far from being known (Haughn and Chaudhury 2005). Recent evidence from *Arabidopsis* suggests that the endosperm may be the primary determinant seed dormancy being sufficient and necessary to confer the coat-dormancy. Ultrastructural changes in the endosperm (e.g. vacuolation, cell wall stretching and weakening or even cell separation) occur only when the coat dormancy has been liberated. These processes are confined at the micropylar level being the vacuolation inhibited by ABA (Bethke et al. 2007). Recent studies showed that putative orthologs of cell-wall remodeling genes (i.e. MAN and cellulases) and proteases are expressed in a complex manner during micropylar endosperm cap weakening, suggesting distinct roles of these genes in the radicle and micropylar endosperm cap (Morris et al. 2011).

A detailed analysis of Fig. 4 suggests that *SoMAN7* plays a notable role in phase I, highlighting its high expression during the first 3 h being this early expression strongly inhibited by nitrate. On the other hand, *SoMAN6* participates in a lower intensity than *SoMAN7* and it is hardly affected by nitrate. This fact did not occur with *SoMAN7* and consequently we can suggest that *SoMAN6* and not *SoMAN7* is involved in the *S. officinale* germination induced by nitrate. Taken together the recent results in *Arabidopsis* (Iglesias-Fernández et al. 2011a,2011b) and *S. officinale*, we can conclude that the nitrate signaling seems to be linked to seed covering softening, being MAN activity a notable target (Fig. 7). Supporting the results of this work, it has recently been demonstrated that *GaMAN1* expression and MAN activity were initiated in the micropylar endosperm but spread to the lateral endosperm (Queiroz et al. 2012). Lee et al. (2012) propose that the MAN degrades the mucilage heteromannan-enriched to produce signaling oligosaccharides which have growth-promoting effects on the germinating seed.

Nitrate alters the expression patterns of *SoNCED6*, *SoNCED9*, *SoCYP707A2* and *SoABI5*

The dormancy maintenance in imbibed seeds requires de novo ABA synthesis (Nambara et al. 2010). However, the ABA involvement during seed imbibition is poorly described compared with its role in seed development (Nambara et al. 2010). One of the main purposes of this research was to know at molecular level if nitrate, whose influence on the germination of some seeds

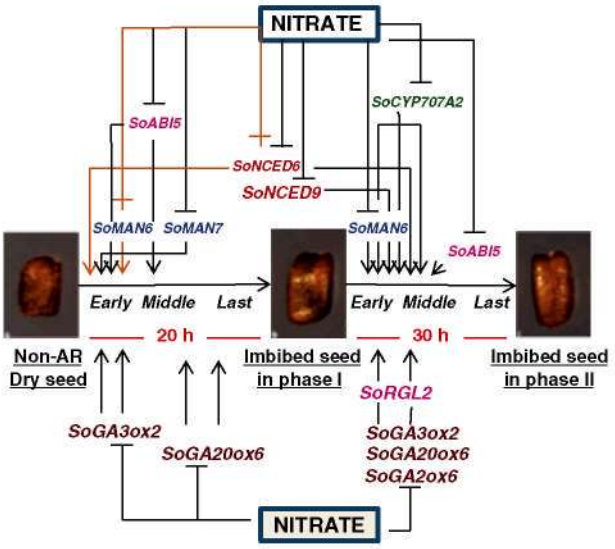

Fig. 7. Changes in the gene expression induced by nitrate during imbibition of *Sisymbrium officinale* non-AR seeds.

such as *S. officinale*, is beyond doubt (Alboresi et al. 2005, Iglesias-Fernández et al. 2011a), alters the gene expression involved in ABA metabolism and signaling during early imbibition. The transcription of *SoNCED6* and *SoNCED9* in absence of nitrate is sharply required at the start of imbibition (3 h) and is differentially affected by nitrate. By contrast, the nitrate markedly inhibited the transcription of *SoNCED6* and *SoNCED9* at the end of imbibition (22–26 h). On the other hand, the data from Fig. 5 clearly demonstrate that the transcription of *SoCYP707A2* is much higher than that of *SoNCED6* and *SoNCED9*, suggesting that a tendency to ABA degradation exists in the absence of nitrate. Even in these circumstances, the nitrate stimulates the early transcription of *SoCYP707A2* and strongly inhibits that of *SoNCED6* and *SoNCED9* mainly between 22 and 26 h. Considering that the expression of both *SoNCED6* and *SoNCED9* concomitantly decreases during the imbibition and the nitrate further reduces these expression patterns, we can conclude from Fig. 5 that nitrate clearly leads to a strong ABA decrease (summarizing in Fig. 7). In this scenario, GAs signaling and probably ethylene as well are promoted and seed is ready to begin germination (Matilla et al. 2005, Iglesias-Fernández and Matilla 2009, Seo et al. 2009). That is, a decrease in ABA sensitivity and increase in GAs sensitivity appear to take place (Linkies and Leubner-Metzger 2012). When ABA synthesis is prevented (aba1 mutant), endosperm weakening and rupture processes may take place (Piskurewicz et al. 2008). After *Arabidopsis* seed imbibition, ABA levels drop rapidly and it is likely that CYP707A hydroxylases are involved in these processes

(Okamoto et al. 2006). Thus, CYP707A2, but not CYP707A1, has shown to play a major role in ABA degradation during early seed imbibition (Okamoto et al. 2006) and to response to exogenous nitrate (Matakiadis et al. 2009). Nitrate also decreases the dormancy level in Cvi dormant seeds (Ali-Rachedi et al. 2004) whereas *A. thaliana* ecotype Columbia seeds broke seed dormancy via changes in ABA sensitivity but not ABA synthesis (Bethke et al. 2006a,2006b, Bethke et al. 2007).

In *Arabidopsis*, ABA stimulates the de novo accumulation of ABI3 and ABI5, which are necessary to repress germination (López-Molina et al. 2001, 2002, Piskurewicz et al. 2008, Lee et al. 2010). It is well known that mature seeds contain high levels of ABA and high amounts of ABI5. However, ABI5 induction is not observed after seed imbibition unless exogenous ABA is applied (López-Molina et al. 2001). ABA triggers ABI5 accumulation and phosphorylation, which is necessary for its activity. More recently, down-regulation of ABI5 levels in response to GAs was reported by Piskurewicz et al. (2008). In this work, nitrate positively alters the *SoABI5* transcript levels when the *SoNCED9* expression is stimulated. The *SoABI5* expression is stimulated by exogenous ABA in *S. officinale* seeds (data not shown). By contrast, at the beginning and the end of imbibition, nitrate clearly inhibited *SoABI5* expression (Fig. 5D), being this effect remarkable when testa rupture reaches 50%. These results agree with those obtained in *SoNCED6*, *SoNCED9* and *SoCYP707A2* expression and reinforce the positive effect of nitrate during imbibition of non-AR *S. officinale* seeds. In summary, we are aware that these *SoABI5* expression data should be confirmed with further experiments due to the fact that the activity of this TF depends not only on ABI5 transcriptional activity but also on the protein level, post-translational changes (i.e. phosphorylation) and a hormonal-regulated network (Piskurewicz et al. 2008, Lee et al. 2010). It must not be forgotten that ABI5 confers seed ABA responsiveness (López-Molina et al. 2001, Finkelstein et al. 2002, Holdsworth et al. 2008b).

Changes in gene expression of *SoGA20ox2*, *SoGA2ox6*, *SoGA3ox2* and *SoRGL2* induced by nitrate

In contrast with ABA, GAs levels are initially very low in dry seeds and rise upon seed imbibition (Yamaguchi and Nambara 2006). The *Arabidopsis* genome contains RGL1 and RGL2, with RGL2 being a regulator of seed germination in response to GAs (Lee et al. 2002, 2010). Summarizing, (1) RGL2 is required for control of ABA content and ABI5 function, (2) RGL2 and ABI5 are positively regulated by ABA and negatively regulated by GAs, with ABI5 acting as a critical checkpoint during germination, (3) low GAs levels result in an elevation of endogenous ABA levels, (4) RGL2 over accumulation is necessary for this response to low GAs levels to take place and (5) increased GAs levels lead to RGL2 degradation, repressing seed germination (Piskurewicz et al. 2008, 2009, Lee et al. 2010). If the nitrate has a positive effect on the ABA reduction in non-AR *S. officinale* imbibing seeds (third section of discussion), *SoRGL2* should be expected to decline in their presence. Indeed, the *SoRGL2* expression tends to decrease after 6 h imbibition in the presence of nitrate. However, although nitrate negatively affect *SoRGL2* expression it is necessary that the expression pattern of genes involved in GAs synthesis and degradation are stimulated and inhibited by nitrate, respectively. This would cause a high GAs/ABA ratio and a RGL2 destruction via the ubiquitin-proteasome 26S pathway after binding to a GAs receptor (GID1), which enhances RGL2 protein interaction with SLY1 (F-boxprotein) (Schwechheimer and Willige 2009). We demonstrated in Figs 6 and 7 that the *SoGA2ox6* expression is strongly inhibited in the control and much more in the presence of nitrate. Taking into account that the nitrate: (1) dramatically inhibits *SoGA3ox2* expression during phase I and slightly after and (2) it decreases *SoGA20ox2* expression, we can suggest that nitrate also provokes the ABA inhibition in non-AR *S. officinale* seeds whereas GAs synthesis is not positively affected. This positive effect of nitrate will be carried out when the *S. officinale* seed acquires the AR status (Iglesias-Fernández and Matilla 2009). That is, when the AR network has been triggered and the favorable germination conditions appear.

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